CHARACTERIZATION OF NUCLEAR AND SATELLITE DNA FROM TRYPANOSOMES[1]

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(Received February 3, 1971)

SUMMARY We studied the DNA of four clones of three trypanosoma species by CsCl density gradient centrifugation and electron-microscopy. We found that although *T. evansi* AK clone has no kinetoplast it contains satellite DNA with the same density as kinetoplast DNA of *T. evansi* K clone. To investigate the *in situ* localization to satellite DNA in *T. evansi* AK clone, we examined thin sections of ^{3}H-thymidine-labeled parasites by electron microscopic radioautography and studied the DNA released from isolated kinetoplast envelopes by electron microscopy. We found that the extra-nuclear DNA is present in the kinetoplast envelope, although extra-nuclear DNA in trypanosoma with kinetoplasts is thought to be concentrated in the kinetonucleus (an inclusion in the kinetoplast). The function of satellite DNA in *T. evansi* AK clone is discussed in relation to the multiplication of AK forms.

Minor circular DNA were found both in the nuclear fraction and the kinetoplast fraction from *T. cruzi*, *T. gambiense* and *T. evansi* K clone. The smallest minor DNA had a contour length of 0.11 μ. We did not find minor circular DNA in preparations from *T. evansi* AK clone.

INTRODUCTION

The kinetoplast is a self-duplicating organelle containing DNA which is peculiar to the family Trypanosomatidae. A number of workers (See Rudozinska and Vickerman, 1968) showed by electron microscopy that the kinetoplast consists of a two-layered envelope membrane (kinetoplast double membrane, kinetoplast envelope) containing a dense fibrous inclusion (kinetonucleus). The kinetoplast is a permanent organelle, but it is possible to obtain organisms devoid of the kinetoplast (hereafter referred to as the AK form) by treating the cells with acriflavine or various other drugs (Werbitzki, 1910). Of the many species of Trypanosomatidae, only one has no kinetoplast, *T. equinum*. AK forms of *T. evansi* and *T. equiperdum*, also appear spontaneously and they are capable of multiplication (Tobie, 1951; Hoare, 1954). Inoki (1956) and Inoki et al. (1960) first proved by the single cell inoculation technique (Inoki, 1960) that artificially and

1 This work was supported in part by a grant in aid from the Ministry of Education, Japan.

spontaneously appearing AK forms of *T. gambiense* can not duplicate, while those of *T. evansi* can. These spontaneously appearing AK forms usually lack the fibrous inclusion, but still retain the envelope membrane (Mühlpfordt, 1963; Inoki and Suganuma, 1964; Rudzinska and Vickerman, 1968). However, Inoki et al. (1969) found that treatment of *T. gambiense* with *p*-Rosaniline resulted in elimination of both the kinetonucleus and kinetoplast envelope. On the other hand, in *T. evansi* only the kinetonucleus disappeared on treatment with *p*-Rosaniline. The difference of the actions of *p*-Rosaniline on the kinetoplasts of these two species is unknown. Many investigators have shown by Feulgen staining and radioautography that DNA is localized in the fibrous inclusion (kinetonucleus) of the kinetoplast.

DNA of the kinetoplast differs from that of the nucleus in buoyant density, forming satellite DNA on ultracentrifugation in a CsCl gradient. The density and GC content of DNA from kinetoplasts are lower than those of nuclear DNA (DuBuy et al., 1965; Riou and Paoletti, 1967; Riou and Pautrizel, 1969).

We studied the DNA of four clones of three species of trypanosomes by CsCl density gradient centrifugation and electron-microscopy. The properties of DNA in akinetoplast trypanosomes capable of multiplication, such as the AK form of *T. evansi*, seem of interest in understanding the contribution of kinetoplast DNA to multiplication. This paper reports results showing that clones of the AK form of *T. evansi* contain satellite DNA although they have no fibrous inclusion (kinetonucleus). The localization of satellite DNA in this strain was studied by electron-microscopic radioautography and release of DNA from the kinetoplast envelope was observed.

MATERIALS AND METHODS

1. *Species of trypanosoma*

Three species of trypanosoma were examined. *T. gambiense*, Wellcome strain was given by courtesy of Dr. Max C. McCowen, the Eli Lilly Research Laboratories, Indiana, U.S.A. and *T. evansi* (K form), Taiwan strain was obtained from the National Institute of Animal Health (Tokyo). The clone of the AK form of *T. evansi* (AK clone) was isolated by Inoki et al. (1961). These strains are maintained in this laboratory by passages through ddo mice. The AK forms usually appear at a level of about 5% in the *T. evansi* K clone and of less than 1% in *T. gambiense*. *T. cruzi* was obtained from N.I.H., Bethesda, U.S.A. and maintained and cultured in a diphasic medium (Taylor and Baker, 1968). AK forms appear at a frequency of less than 1% in *in vitro* cultures.

2. *Labeling of cells with ^{3}H-thymidine*

When parasites had reached a level of 10^9/ml in the blood stream of ddo mice, 3 to 4 days after intraperitoneal inoculation, the blood was collected in 0.5% glucose–0.5% citrate–0.5% saline solution from the heart after opening the thorax under chloroform anesthesia. The trypanosomes were separated from blood elements by repeated differential centrifugation. To check the contamination of the preparation of trypanosomes with blood elements, part of the preparation was stained with Giemsa solution and examined microscopically. If contamination was not appreciable, the trypanosome cells were suspended in a mixture of 6 ml of glucose–citrate–saline solution and 4 ml of the liquid phase of the diphasic medium for *T. cruzi*. ^{3}H-Thymidine was added at a final concentration of 10 μc/ml to the suspension and the mixture was incubated for 5 hr at 37 C. To label *T. cruzi*, parasites were grown in 100 ml flasks containing 10 ml of the liquid medium of Boné and Parent (1963) in the presence per ml of 200 μg streptomycin, 200 unit of penicillin and 5 μc of ^{3}H-thymidine for 10 days at 25 C.

3. *Preparation of DNA*

DNA was extracted from whole cells, kinetoplasts and nuclei using the phenol procedure of Kirby (1957).

Kinetoplast DNA was prepared by the technique used for its isolation from *Leishmania enriettii* (DuBuy et al., 1965). The parasites were suspended in 0.85% saline solution, and the nonkinetoplast DNA was digested and removed by incubation with DNase (25 μg/ml, final concentration) for 30 min at 37 C.

Nuclei were separated from kinetoplasts and other cell components as follows. Cells were suspended in distilled water and ruptured by subjection to osmotic shock for one hour. The cell components were collected by centrifugation at 7,000 rpm for 30 min and resuspended in 0.25 M sucrose–1% albumin–2% Triton X-100 solution. The suspension was homogenized for 30 min in an ice-bath and then flagella were sheared off using a Waring Blender. Then the nuclei were separated from kinetoplasts and other cell elements by centrifugation of 3,000 rpm for 10 min. The pellet was suspended in SSC and DNA was isolated from the suspension as nuclear DNA.

This method was unsatisfactory for isolation of the nuclei of *T. cruzi* from kinetoplasts and other cell components because the nucleus was located close to the kinetoplast and was almost the same size as the latter. Therefore, DNA extracted from the akinetoplastic form induced with acriflavine was used as nuclear DNA. The akinetoplastic cells were obtained by culturing *T. cruzi* with 0.2 μg/ml of acriflavine. AK forms appeared at a level of about 80% and other cells had small kinetoplasts.

The whole cells, nuclei and kinetoplasts prepared in this way were suspended in SSC and lyzed by treatment with 0.8% sodium lauroyl sarcosinate (SLS) at 60 C for 30 min. The lysates were treated with pronase-p (final concentration 1 mg/ml) at 37 C for 15 hr, and DNA was extracted by the phenol procedure. The phenol in the DNA solution was removed by dialysis for 2 days against three changes of SSC at 4 C.

4. *Fractionation of DNA by CsCl density gradient centrifugation*

CsCl density gradient centrifugation was carried out by the method of Meselson et al. (1957). ^{14}C-labeled DNA of *Micrococcus lysodeikticus* (ρ=1.731 g/ml) was used as a density marker. DNA was fractionated by ultracentrifugation at 36,000 rpm for about 48 hr at 10 C using a Beckman SW 50 rotor. To obtain good separation of satellite DNA from the main DNA, whole cell DNA was centrifuged in a No. 40 angle rotor.

5. *Electron microscopic observation of DNA*

The samples of the main and satellite fractions of DNA separated by CsCl gradient centrifugation were suspended in 2 M ammonium acetate solution. Cytochrome c was then added at a final concentration of 0.03% and the mixture was immediately spread on a water surface by the method of Kleinschmidt et al. (1962). The DNA molecules were successively shadowed with platinum-paladium at an angle of 6°, rotating the specimen. The specimens were examined in Hitachi 11-B electron microscope, and the lengths of the DNA molecules were measured with a map measure at a final magnification of 42,000.

6. *Observation of DNA released from ruptured kinetoplast envelopes of T. evansi AK clone*

Kinetoplast envelopes of cells of the *T. evansi* AK clone were prepared by the same technique used for isolation of kinetoplasts from *T. gambiense* (Ozeki et al., 1970). Envelopes were ruptured by osmotic shock and examined by electron microscopy.

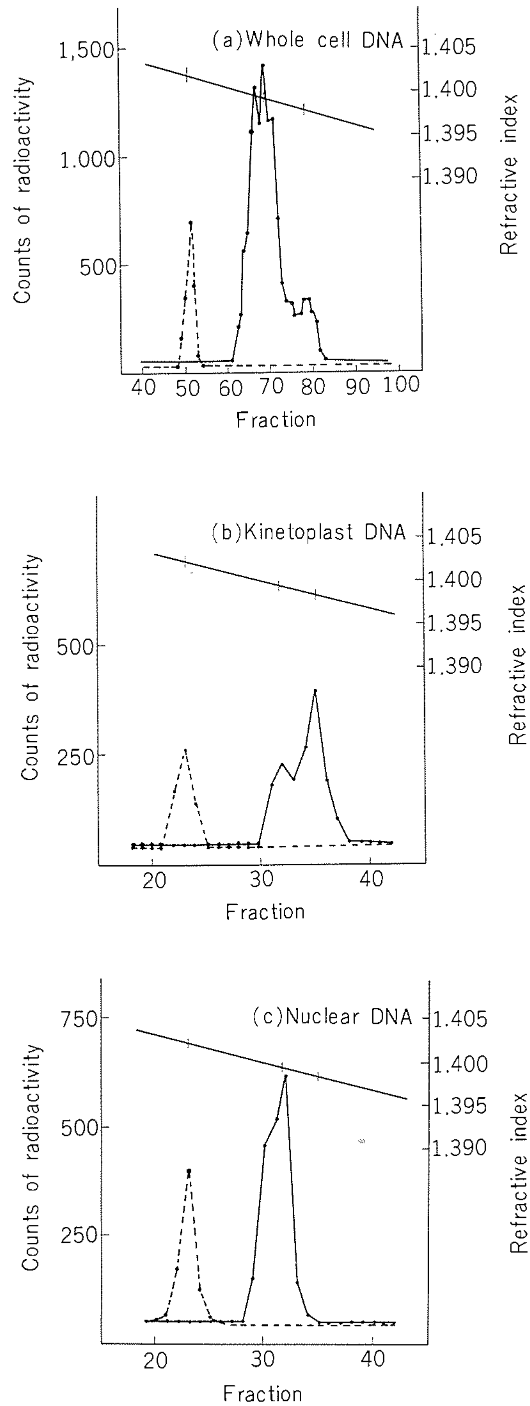
7. *Electron microscopic radioautography*

Trypanosome cells labeled with ^{3}H-thymidine were fixed at 4 C for one hour in 0.013 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and washed for one hour with 0.013 M phosphate buffer containing 0.25 M sucrose. The materials were post-fixed at 4 C for one hour with 1.5% osmium tetroxide in isotonic buffer. After dehydration, materials were embedded and stained as described previously (Inoki and Ozeki, 1969). Specimens were prepared for radioautography by the method of Ozeki et al. (1971). Sakura NRH_2 Emulsion was used to detect radioactivity in trypanosome cells. After three weeks exposure, the radioautographs were developed for 5 min in Sakura Konidol × and fixed for 5 min in Kodak Fixative.

RESULTS

1. *Analysis of DNA by CsCl density gradient centrifugation*

On CsCl density gradient centrifugation of whole cell DNA a main band and one satellite band were obtained. (Figs. 1a, 2a, 3a, 4 and Table 1). The main band had a buoyant density of ρ=1.703 g/ml in *T. gambiense*, ρ=1.709 g/ml in *T. cruzi*, ρ=1.704 g/ml in *T. evansi* K clone and ρ=1.704 g/ml in *T. evansi* AK clone. The main band seemed to be nuclear DNA, judging from the results in Fig. 1c and 2c on DNA extracted from nuclei of AK forms induced by acriflavine. DNA from isolated nuclei of *T. evansi* K clone and *T. evansi*



▶ FIGURE 1. *Analysis of T. gambiense DNA by CsCl density gradient centrifugation.*
○——○ *T. gambiense DNA.*
○-------○ *Micrococcus lysodeikticus DNA* (ρ= *1.731 g/ml) as a density marker.*
(*a*) *whole cell DNA.* (*b*) *kinetoplast DNA.*
(*c*) *nuclear DNA*

AK clone were not examined.

The satellite band had a buoyant density of ρ=1.688 g/ml in *T. gambiense*, ρ=1.699 g/ml in *T. cruzi* and ρ=1.692 g/ml in *T. evansi* K clone and seemed to be kinetoplast DNA, as DNA extracted from DNase-treated cells had the same density (Fig. 1b, 2b and 3b). Riou and Pautrizel (1969) reported the density of DNA in *T. gambiense* and *T. cruzi* measured by densitometer tracing of UV-absorbance at 260 mμ on CsCl analytical ultracentrifugation. Our results on the density and number of satellite band agree with their results for *T. cruzi*, but not for *T. gambiense*. Riou and Pautrizel found two satellite DNA's with buoyant densities of 1.701 g/ml and 1.690 g/ml in *T. gambiense*. However, we found only one satellite DNA of ρ=1.688 g/ml in this trypanosome. Our results are consistent with previous reports that satellite DNA represents kinetoplast DNA (DuBuy et al., 1965; Riou and Pautrizel, 1969). In our experiments, however, satellite DNA with a buoyant density of ρ=1.693 g/ml was also found in *T. evansi* AK clone (Fig. 4). This result is in contrast to those obtained on drug-induced AK forms of *T. cruzi* and other trypanosomes. The latter forms do not have satellite DNA (See Fig. 2c; Steinert and Van Assel, 1967; Simpson, 1968). To confirm the existence of satellite DNA in cells of the AK clone of *T. evansi*, a double-labeling experiment was carried out. Whole cell DNA labeled with ^{14}C-thymidine was mixed with the main DNA labeled with ^{3}H-thymidine, which had been isolated by CsCl gradient centrifugation. The mixture was resubjected to CsCl gradient centrifugation. Fig. 5 shows clearly that radioactivity of ^{14}C appeared in the position of satellite

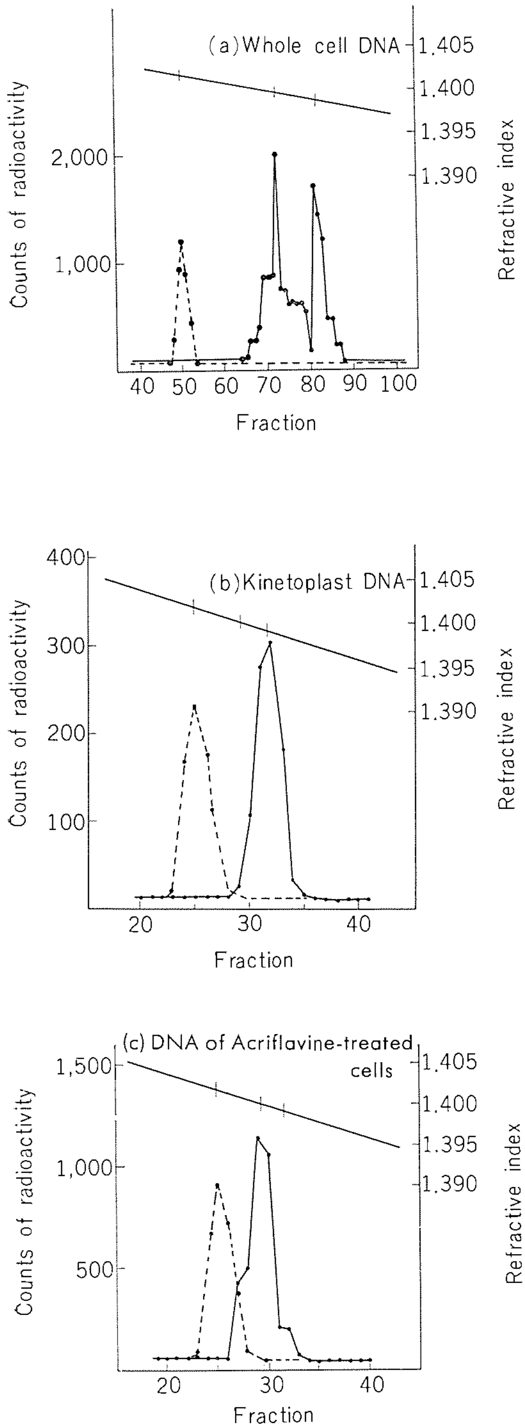


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FIGURE 2. *Analysis of T. cruzi DNA by CsCl density gradient centrifugation.*
○——○ *T. cruzi DNA.*
○-------○ *Micrococcus lysodeikticus DNA* (ρ= *1.731 g/ml*).
(*a*) *whole cell DNA* (*b*) *kinetoplast DNA*
(*c*) *DNA from acriflavine treated cells.*

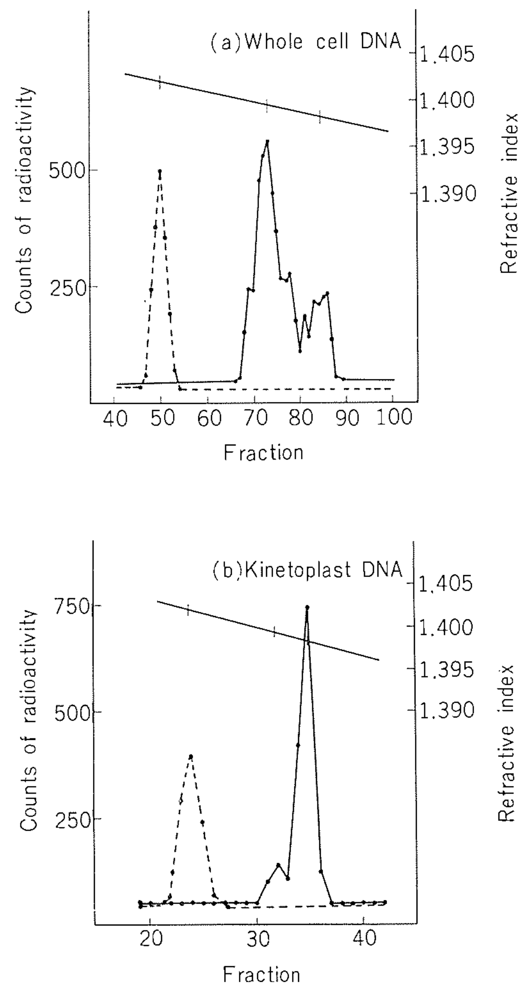

FIGURE 3. *Analysis of DNA of T. evansi K clone by CsCl density gradient centrifugation.*
○——○ *T. evansi K clone DNA.*
○-------○ *Micrococcus lysodeikticus DNA* (ρ= *1.731 g/ml*).
(*a*) *whole cell DNA* (*b*) *kinetoplast DNA*

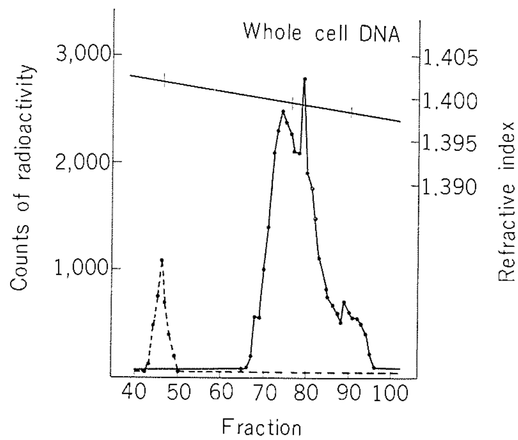

FIGURE 4. *Analysis of DNA of T. evansi AK clone by CsCl density gradient centrifugation.*
○——○ *Whole cell DNA of T. evansi AK clone.*
○-------○ *Micrococcus lysodeikticus DNA* (ρ=*1.731 g/ml*).

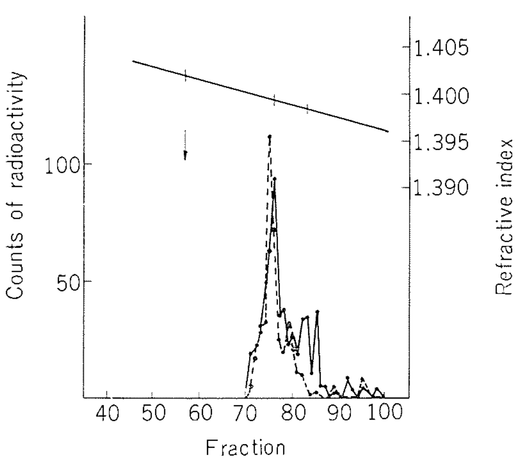

FIGURE 5. *Comparison of the patterns on CsCl gradient centrifugation of whole cell DNA of T. evansi AK clone and that of purified, main DNA.*
○——○ *Whole cell DNA labeled with* ^{14}*C-thymidine.*
○-------○ ^{3}*H-labeled main DNA.*
The arrow shows the position of Micrococcus lysodeikticus DNA (ρ=*1.731 g/ml*).

TABLE 1. *Buoyant density of the main and satellite bands on CsCl density gradient centrifugation*

Species of trypanosoma	Type of DNA	Buoyant density
T. gambiense	nuclear	1.703
	satellite	1.688
T. cruzi	nuclear	1.709
	satellite	1.699
T. evansi K clone	nuclear	1.704
	satellite	1.692
T. evansi AK clone	nuclear	1.704
	satellite	1.693

DNA, while that of ^{3}H did not. These results suggest that the *T. evansi* AK clone used has satellite DNA. Unlike the kinetoplast DNA of other trypanosoma the satellite DNA in *T. evansi* AK clone could not be isolated by DNase treatment.

2. *Electron microscopic observation of DNA molecules*

Electron microscopic observations were made of the DNA molecules in the main and satellite fractions of *T cruzi*, *T. gambiense*, *T. evansi* K clone and AK done obtained by CsCl density gradient centrifugation. Long, linear DNA molecules were observed in both the nuclear fraction and kinetoplast or satellite fraction. The actual length of linear DNA could not be estimated owing to breakage, but the linear DNA molecules in the nuclear fraction were longer than those in the kinetoplast fraction. The molecules of DNA in the nuclear fraction of *T. evansi* AK clone were also longer than those in the satellite fraction. The length of molecules of satellite DNA of *T. evansi* AK clone was similar to that of molecules of kinetoplast DNA of *T. cruzi* and *T. gambiense*.

Minor circular DNA molecules were found both in the nuclear fraction and in the kinetoplast fraction obtained by CsCl density gradient centrifugation of DNA from *T. cruzi*, *T.*

TABLE 2. *Frequency distribution of the lengths of circular DNA molecules in T. cruzi and T. gambiense*

Length (μ)	Species	
	T. cruzi	*T. gambiense*
0.10– 0.19	5	0
0.20– 0.29	61	22
0.30– 0.39	17	4
0.40– 0.49	20	2
0.50– 0.59	0	2
0.60– 0.79	4	4
0.80– 0.99	4	6
1.00– 1.19	2	6
1.20– 1.39	2	9
1.40– 1.59	6	2
1.60– 1.79	16	0
1.80– 1.99	4	0
2.00– 2.49	2	0
2.50– 2.99	4	0
3.00–15.00	6	3
Total	153	60

gambiense and *T. evansi* K clone. We did not find minor circular DNA in preparations from *T. evansi* AK clone. Table 2 shows the lengths of the minor circular DNA in the nuclear and kinetoplast fractions of *T. cruzi* and *T. gambiense*. The lengths of that from *T. cruzi* were mainly between 0.2 and 0.5 μ and between 1.4 and 1.8 μ. Minor circular DNA molecules were more frequently found in preparations of DNA from *T. cruzi* than in those from *T. gambiense*. The shortest circular DNA molecules were 0.11 μ long in *T. cruzi* and 0.21 μ in *T. gambiense*, and the longest were 12 μ long in *T. cruzi* and 15 μ in *T. gambiense*. Fig. 6a shows electron micrographs of two linear DNA molecules from *T. evansi* AK clone. Fig. 6b shows two minor circular molecules and one linear molecule from the kinetoplast DNA fraction of *T. cruzi*.

3. *Studies on the localization of DNA in T. evansi AK clone*

To investigate the *in situ* localization of satellite DNA in *T evansi* AK clone, electron microscopic radioautography was carried out. ^{3}H-Thymidine was incorporated into the kinetoplast envelope as well as the nucleus, but not into the electron-dense fragment inside the envelope (Fig. 7a, 7b, 7c). This fragment has been considered to be the remains of the kineto nucleus. This result suggests that the satellite DNA of *T. evansi* AK clone is located inside the kinetoplast envelope, although the AK clone has no kinetonucleus. Satellite DNA in other trypanosomes with a kinetoplast is found in the kinetonucleus (fibrous inclusion) itself (Riou and Pautrizel, 1969). Ozeki et al. (1971) showed that the silver grains of ^{3}H-thymidine mainly appeared in the nucleus, kinetonucleus and blepharoplast, but not inside of kinetoplast envelope of *T. cruzi*.

Next we tried to find DNA in the kinetoplast envelope of *T. evansi* AK clone by electron microscopy. Kinetoplast envelopes were prepared as described under isolation of kinetoplasts in the Materials and Methods and were ruptured by osmotic shock. Fig. 8 shows an electron micrograph of DNA released from the kinetoplast envelope which is still attached to the basal portion of the flagellum, so that the possibility that it is contaminating nuclear DNA is very unlikely. In addition to DNA molecules, a large number of fragments of kinetoplast envelope are seen dispersed about or intermingling with the DNA molecules. These fragments were seen near flagella, but not in other portions of the grid.

DISCUSSION

We studied the DNA of three species of trypanosome by CsCl density gradient centrifugation, and confirmed that trypanosomes with kinetoplasts have satellite DNA, as reported by other investigators (Steinert and Van Assel, 1967; Simpson, 1968; Riou and Pautrizel, 1969; Renger and Wolstenholme, 1970). Some of these workers also showed that whole cell DNA extracted from AK forms of *T. mega*, *Leish-*

mania tarentolae and *T. lewisi* induced by acriflavine have no band of satellite DNA in CsCl analytical centrifugation and that the latter band is kinetoplast DNA. In this work, also CsCl density gradient centrifugation of DNA of the akinetoplastic form of *T. cruzi* gave no satellite DNA (Fig. 2c). Thus, it seems that satellite DNA is indeed kinetoplast DNA. However, we found that although *T. evansi* AK clone has no kinetoplast, it contains satellite DNA with the same density as kinetoplast DNA of *T. evansi* K clone (Fig. 4).

The following two possibilities with regard to the existence of satellite DNA in the AK form of *T. evansi* seem unlikely. (1) The existence of satellite DNA in the AK clone of *T. evansi* is due to blood stream form. AK forms of *T. evansi* are not cultured forms such as those of *T. cruzi*, *T. mega*, *Leishmania tarentolae* and *T. lewisi*, but blood stream forms. However, we found that when the blood stream form of *T. gambiense* was treated with acriflavine the satellite band disappeared (unpublished). (2) The AK forms appear spontaneously in low frequency in trypanosome species, such as *T. cruzi*, *T. gambiense* and *T. mega*, but these AK forms can not multiply. Therefore, we could not see whether these spontaneous AK forms had satellite DNA. No satellite DNA was found in artificially induced AK forms of these trypanosomes by CsCl density gradient centrifugation. Therefore, the second possibility is that satellite DNA may be eliminated during the treatment inducing AK forms. However, the satellite DNA of *T. evansi* AK clone did not disappear on treatment with acriflavine (unpublished). Thus, these two possibility may be excluded. The existence of satellite DNA in the AK clone of *T. evansi* may be a particular character of this species. The AK forms of this species could multiply, whereas those of *T. cruzi*, *T. gambiense* and *T. mega* could not. It seems likely that satellite DNA may also be present in the AK forms of *T. equinum* and *T. equiperdum* which can multiply.

Electron microscopic studies showed that the satellite DNA in *T. evansi* AK clone does not have a definite structure, such as the fibrous inclusion (kinetonucleus) in trypanosomes with a kinetoplast, but is present in the kinetoplast envelope and widely distributed within the envelope. However, we have not yet tested whether DNA released from the kinetoplast envelope forms satellite DNA on CsCl gradient centrifugation. The kinetoplast is generally thought to be a mitochondrion of trypanosomes (Clark and Wallace, 1960; Steinert, 1960). DNA of the kinetoplast is usually concentrated in a definite structure, the kinetonucleus, whereas DNA of mitochondria in other organisms seems to be dispersed inside the mitochondrial membrane. The satellite DNA of the AK clone of *T. evansi* seems to be similar to mitochondrial DNA in its intracellular localization. If kinetoplast DNA functions as mitochondrial DNA, its existence must be essential for multiplication of trypanosome species. Our investigations revealed that satellite DNA existed in AK forms which can multiply, but not in those which can not. This suggests that satellite DNA is important for multiplication of trypanosome species.

No morphological difference of kinetoplast, kinetonucleus and its envelope between *T. gambiense* and *T. evansi* could be detected using standard microscopic or electron microscopic

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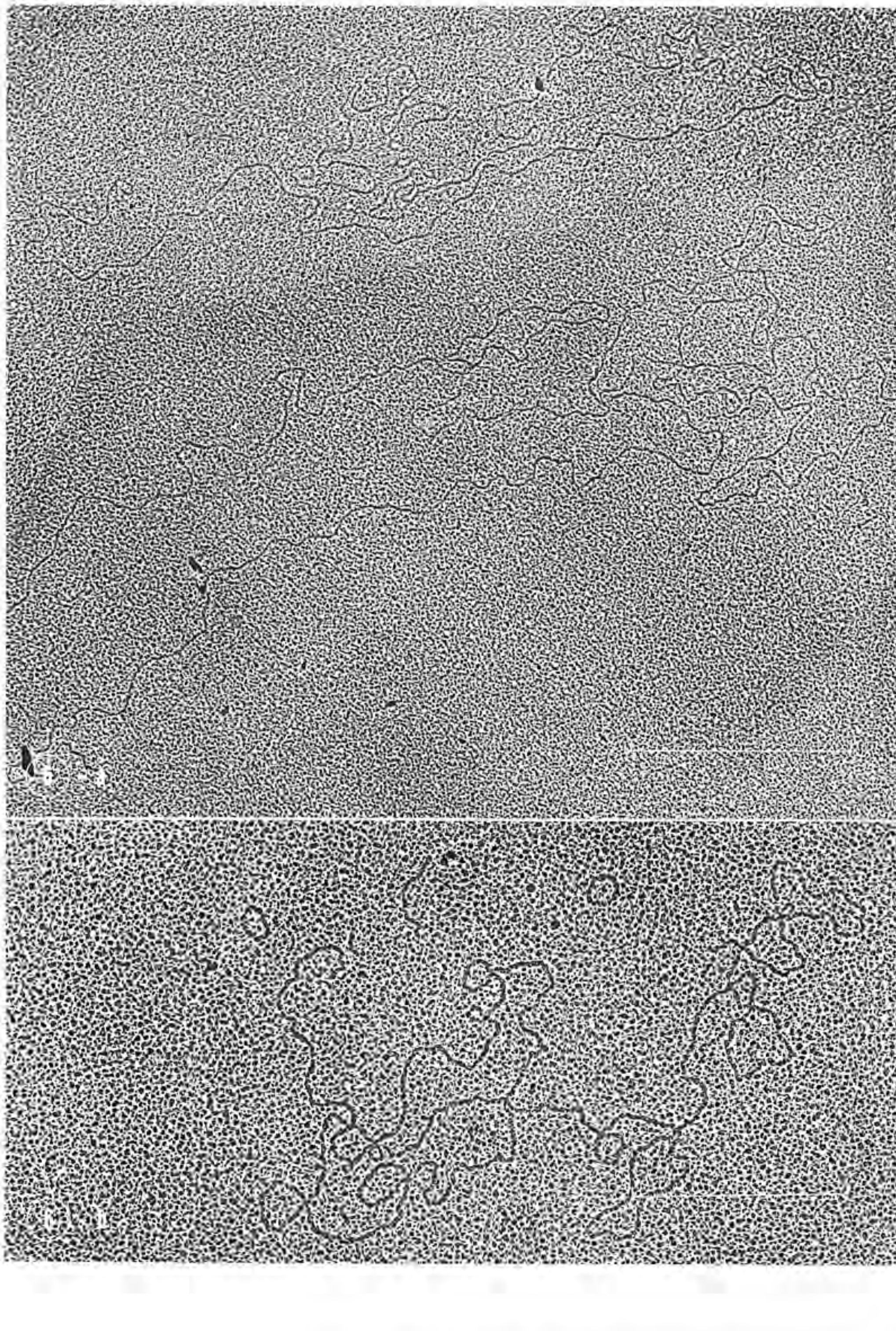
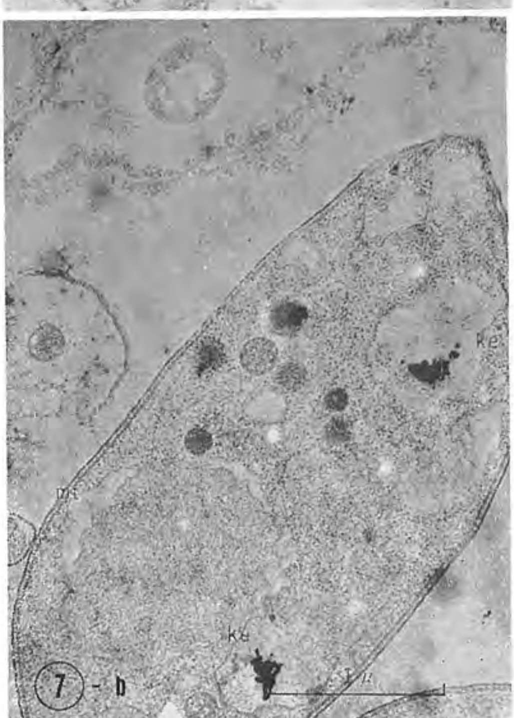
FIGURE 6. *Electron micrographs of satellite DNA or the kinetoplast DNA fraction by CsCl density gradient centrifugation.*

(*a*) *two molecules in the satellite DNA fraction of T. evansi AK clone.*

(*b*) *two minor circular molecules and one linear molecule in the kinetoplast DNA fraction of T. cruzi.*





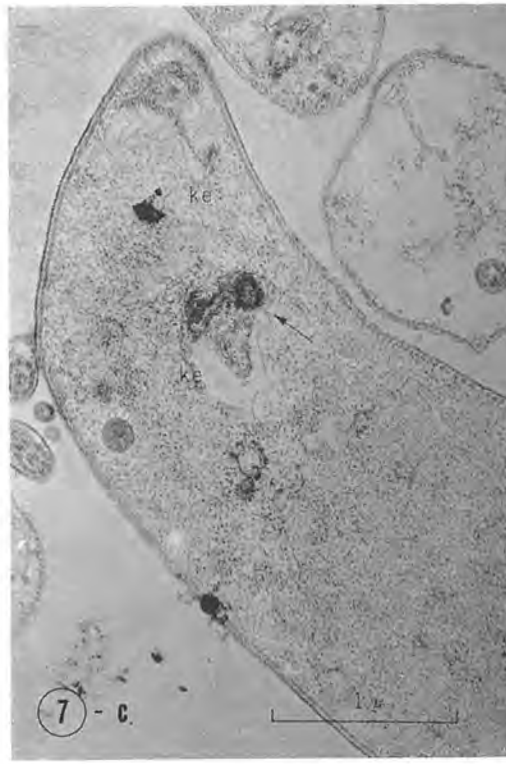


FIGURE 7. *Autoradiographs of T. evansi AK clone incubated with ^{3}H-thymidine (10 μc/ml) for 5 hr.*

H^{3}-thymidine is incorporated into the kinetoplast envelope (Fig. 7b) as well as the nucleus (Fig. 7a), but not into the electron-dense fragment inside the envelope (Fig. 6c). The arrow shows the electron-dense fragment. N, nucleus; Ke, kinetoplast envelope, mt, microtubules.

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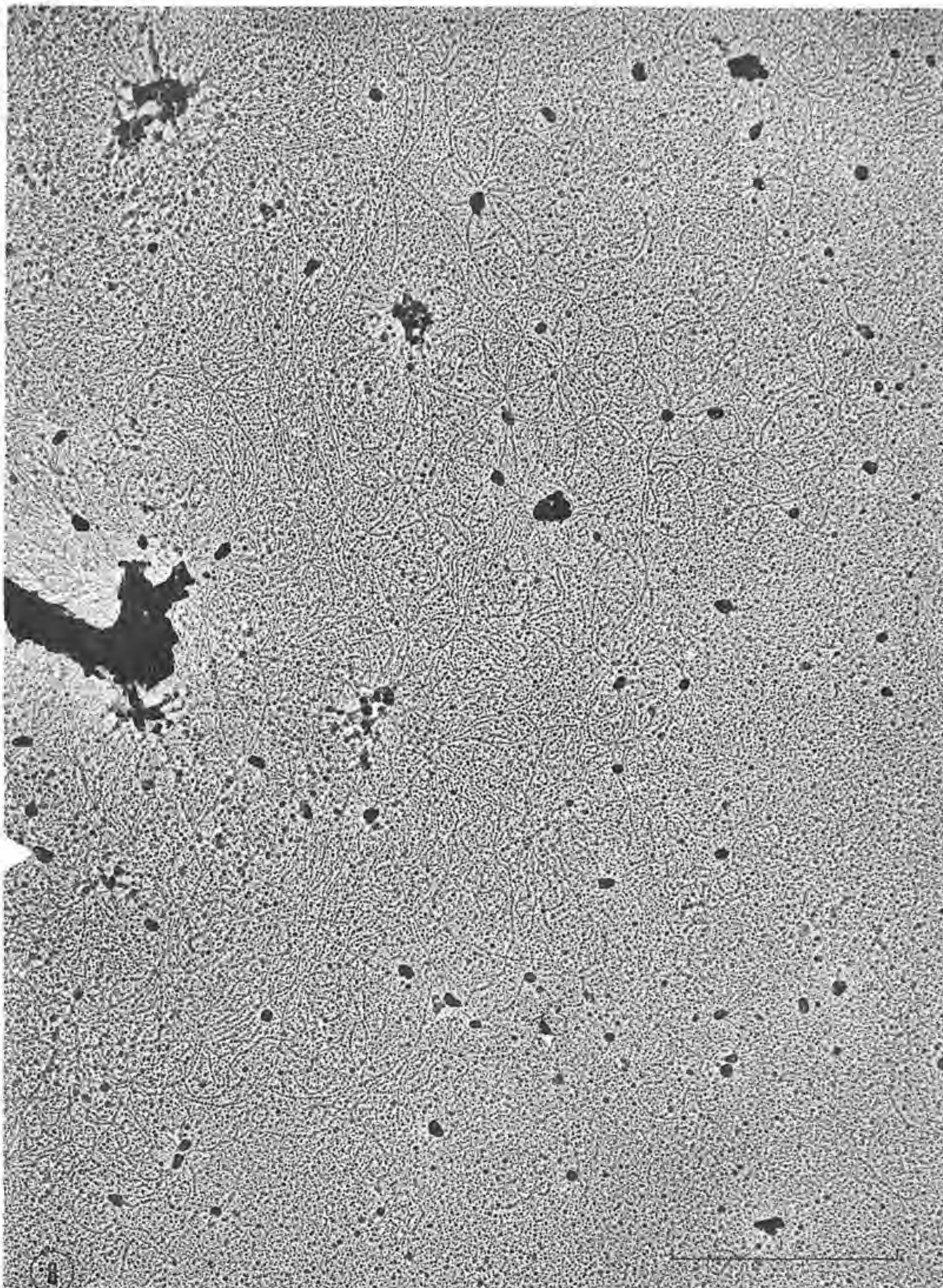
FIGURE 8. *Electron micrograph of DNA released from the kinetoplast envelope of T. evansi AK clone. DNA threads are still attached to the basal body of the flagellum.*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procedures. However, Inoki et al. (1969) showed by electron microscopy that *p*-Rosaniline had different effects on the kinetoplasts of *T. gambiense* and *T. evansi*. They found that treatment of *T. gambiense* with *p*-Rosaniline resulted in elimination of both the fibrous inclusion and kinetoplast envelope. On the other hand, in *T. evansi* only the inclusion disappeared. These results suggest that there is some structural difference between the envelope membranes of the kinetoplasts of these species. This difference might be related to the persistence of satellite DNA when the AK form appears spontaneously or is induced artificially.

Riou and Delain (1969) and Renger and Wolstenholme (1970) reported that the kinetoplast DNA extracted from *T. cruzi* and *T. lewisi* was mainly in the form of minor circular molecules. These minor circular molecules were about 0.45 μ long in *T. cruzi* (Riou and Delain, 1969) and 0.40 μ long in *T. lewisi* (Renger and Wolstenholme, 1970). In our experiments, minor circular molecules were found in the nuclear fraction as well as the kinetoplast fraction on CsCl density gradient centrifugation of DNA of *T. cruzi*, *T. gambiense* and *T. evansi* K clone. The lengths of minor circular molecules of *T. cruzi* were mainly between 0.2 and 0.5 μ and between 1.4 and 1.8 μ. However, DNA in the satellite fraction (kinetoplast fraction) obtained by CsCl density gradient centrifugation consisted mainly of long, linear DNA molecules. Electron micrographs of DNA released from isolated kinetoplast envelopes are similar to those of DNA released from the kinetoplast of *T. gambiense* (Ozeki et al., 1970) or *T. mega* (Laurent and Steinert, 1970) which contain mainly long, linear DNA molecules. These results suggest that there are two different kinds of DNA in the kinetoplast, but minor, circular DNA molecules do not seem to be major components of the kinetoplast or kinetonucleus.

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